THE INHIBITORY EFFECT OF SUCROSE-SORBOSE-AGAR MEDIUM ON A MUTANT OF *NEUROSPORA CRASSA*

by

TRUDY BRAWNER

B. S., Kansas State University, 1959

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

GENETICS

Department of Agronomy

KANSAS STATE UNIVERSITY
Manhatten, Kansas

Thad H Pittenger

1962

TABLE OF CONTENTS

INTRODUCTION....1

MATERIALS AND METHODS....5

RESULTS....8

The Inhibitory Effect....9

The Sucrose Effect....9

The Sorbose Effect....10

The Agar Effect....14

Agar....14

Dialysis and Chromatography....14

Conditions Which Simulate the Agar Effect....19

Sucrose....23

The Influence of Sucrose Hydrolytic Products on the Inhibitory Effect....24

Effects of Sorbose, Salts, and Agar on Sucrose Hydrolysis....27

Inhibitory Substance....30

Characteristics and Inheritance Studies....34

DISCUSSION AND CONCLUSIONS....37

ACKNOWLEDGMENT....50

REFERENCES....51-52

INTRODUCTION

One of the most useful technical advances in the routine experimental use of Neurospora was made by Tatum, Barratt, and Cutter (1949), who were able to demonstrate that Neurospora could be induced to grow in the form of a colony by supplementing the culture medium with 0.1 percent sucrose and one percent sorbose. This method of handling Neurospora has enabled the investigator to utilize many bacteriological plating methods in a variety of experimental approaches to different problems never before possible with Neurospora. Although the colonizing properties of sorbose adversely affect the normal growth of the organism, under appropriate conditions most strains maintain a very high degree of viability when conidia are plated on a medium containing sorbose.

Recently in a routine attempt to recover inositol-requiring heterokaryon compatible isolates from the progeny of a cross of inos a x nic-1 al-2 A, it was noted that approximately half of the inositol-requiring progeny of the cross accumulated a dark brown pigment in the culture medium, while the remaining half did not. Subsequent tests of these cultures revealed that those isolates with the brown accumulation failed to form colonies when the conidia were plated on medium containing sorbose and sucrose, while the normal-appearing isolates produced conidial colonies in the expected numbers. The nicotinic acid-requiring isolates from the cross did not show the brown accumulation, but these strains when plated also segregated for the ability to form colonies on sorbose-supplemented medium. This inability of the conidia of

certain strains to germinate and form colonies on sorbose media had never before been encountered in this laboratory. The segregation of the characteristic among the progeny of the cross suggested that it had a genetic basis.

It has been proposed with convincing evidence (de Serres, et al., 1962) that the inability of wild type 74A conidia to form colonies on medium containing one percent sorbose and 0.1 percent sucrose is positively correlated with the length of time the medium has been autoclaved, the conidial colony-forming ability being in fact a reflection of the degree to which sucrose has been hydrolyzed to glucose and fructose. The autoclaving time routinely utilized in this laboratory is sufficient to obtain good conidial recovery of the nutritionally-deficient and wild type strains regularly studied. With de Serres' work in mind, isolates from the afore-mentioned cross were plated on sorbose medium supplemented with glucose. Those mutant strains incapable of forming conidial colonies on sorbose medium containing 0.1 percent sucrose were fully capable of forming the expected number of conidial colonies on sorbose medium supplemented with 0.02 percent glucose. Exploratory experiments designed to determine what particular component of the sucrose-sorbose medium had an inhibitory effect on these mutant strains revealed a variety of experimental conditions that affected the colony-forming ability of both mutant and standard wild type strains. The sensitivity of the mutant strains was such that it permitted the detection of a whole set of variables never previously suspected of affect-

ing colony-forming ability.

It is important to bear in mind throughout the presentation that this study deals principally with a mutant strain characterized by an impaired or totally lacking function for the utilization of the usual one percent sorbose, 0.1 percent sucrose plating medium. The inhibitory effect, defined as the inability of a strain to produce conidial colonies on sucrose-sorbose-agar medium, has been found to be compounded of the three principal ingredients of the plating medium: sucrose, sorbose, and agar. All must be autoclaved together at the prescribed levels to effect inhibition. Alteration of the levels of sucrose, sorbose, or agar in the proper direction can relieve inhibition of the mutant form. It has also been observed that separate autoclaving of certain of these ingredients or the complete elimination of certain of them from the medium can greatly increase the colony-forming efficiency of the mutant strain. Agar has been reduced to its basic inhibitory condition by dialysis and chromatographic separation of the components. Comparison of mutant reactions to three different media, Westergaard's, Fries' and Vogel's, and an explanation for the unexpected differences in colony-forming efficiency on these three media have been presented. The effect of saturating the inhibitory medium with living conidial and mycelial material with the subsequent relief of inhibition of the mutant strain has been illustrated. All of these considerations have some influence on the basic mutant inhibitory reaction to sucrose-sorbose-agar medium.

The basic problem concerns the inability of the mutant form to utilize normal one percent sorbose, 0.1 percent sucrose, two percent agar medium. The resolution of this problem apparently lies in one of two realms: 1) either inhibition of the mutant strain is due to non-availability of sufficient utilizable carbon sources, as a result of sucrose hydrolysis products' being unavailable or being produced at insufficient levels, or 2) an inhibitory product is formed during autoclaving of the medium which prevents the mutant's utilization of available carbon sources. The reader is entreated to bear in mind that neither of the above conditions applies noticeably to a variety of standard wild type or nutritionally-deficient strains. Only the mutant type evidences this particular sensitivity. Throughout this presentation an attempt will be made to compare the reactions of the mutant strain to the reactions of normal strains. It will be emphasized that a strain carrying the mutation is simply a more sensitive indicator of sub-maximal media conditions, and that the normal strain is often also affected, but to a much lesser degree, by the same adverse conditions.

A somewhat analogous inhibition has been reported with poliovirus (Takemori and Nomura, 1960) and EMC virus (Takemoto and Liebhaber, 1961). Two different viral classes have been described, one class sensitive (morphologically) to agar overlay, and one class unaffected (or even stimulated) by agar medium. The overall effect is comparable to the present Neurospora inhibitory effect. Dissimilarities will be noted in the discussion.

MATERIALS AND METHODS

The medium most extensively employed in this study is commonly referred to as Westergaard's synthetic medium (Westergaard and Mitchell, 1947) and was prepared by adding the following chemical components to one liter of distilled water: 1 g. KNO_3, 1 g. KH_2PO_4, 0.5 g. $MgSO_4 \cdot 7H_2O$, 0.1 g. NaCl, 0.1 g. $CaCl_2$, 10 μg. biotin, and approximately 20 mg. mineral trace elements in solution. Other media used were Fries' synthetic medium (Horowitz and Beadle, 1943) and Vogel's synthetic medium (Vogel, 1956). The standard medium, henceforth referred to as sucrose-sorbose-agar or glucose-sorbose-agar medium, was prepared by supplementing the basal salts solution of Westergaard with five μg. inositol per ml., one percent sorbose (Pfanstiehl Chemical Co., Lot No. 4567), 0.1 percent sucrose or 0.02 percent glucose, and two percent agar (Difco Bacto-Agar, Control Nos. 452452 and 454376), and autoclaving all components together at 18 lb. pressure for 15 minutes, although many variations from this standard will be noted. To effect separation of components during autoclaving, various portions of the medium were prepared double-strength, autoclaved separately, and combined after autoclaving. In the tables and manuscript, brackets around certain components of the medium indicate that these were autoclaved separately and then combined. For example, (agar + sorbose) + (salts + sucrose) indicates that a four percent agar and two percent sorbose solution was autoclaved separately from a double-strength salts and 0.2 percent sucrose solution; the two solutions were then combin-

ed after autoclaving to return the concentration to normal. Unless otherwise stated media were autoclaved at 18 lb. pressure for 15 minutes. In all cases, plating of conidia was accomplished by expelling one ml. of conidial suspension into two ml. of melted overplating medium (one percent agar in distilled water), and overlayering this on solid agar test medium (approximately 20 ml.) in petri dishes. Plates were incubated four to five days at 30°C before scoring. Routinely media were prepared in 100-ml. lots. From this five plates were poured, two of which were overplated with a conidial suspension of the control strain *inos-9*, and three with a conidial suspension of the mutant strain *inos-8*.

The principal strains utilized in this study were obtained by crossing a *nic-1*, *al-2* *A* strain to an *inos* *a* isolate procured from Dr. Laura Garnjobst. Other strains used were *pan-1* *al-1* *24A* and *pan-2* *ad-8* *5A*. The *pan-1* allele used in these studies was 5531, the *nic-1* allele was 3416, the *inos* allele was 37401, and *al-1* and *al-2* were 4637T and 15300, respectively (Barratt, Newmeyer, Perkins and Garnjobst, 1954). The *pan-2* allele was isolated and described by Case and Giles (1958), and the *ad-8* allele was isolated and described by Ishikawa (1960).

Agar dialysate was prepared by dialyzing ten g. moistened agar against 250 ml. distilled water for 24 hours. The dialysate was concentrated to the indicated levels by evaporation, either at 60°C or 100°C. Agar extracts were prepared by freezing the solidified agar overnight, thawing at room temperature, and expelling the liquid by straining the agar through a triple layer of cheesecloth while exerting a small amount of pressure. Agar

was washed by adding about 50X amount of water, allowing agar to settle, decanting the liquid supernatent, adding more water, and repeating the process a minimum of 20 times. The last five washings were done with distilled water. Supernatent from washed agar refers to the liquid decanted from the first washing.

Initially, hemocytometer counts were made to determine the numbers of conidia plated. It was soon discovered that in each experiment nearly 100 percent of the conidia plated formed visible colonies under at least one condition of variability employed in preparation of the plating medium, so hemocytometer determinations were discontinued. The colony-forming efficiency of a strain is defined as the proportion of conidia capable of forming visible colonies on a test medium compared to the number of colonies formed under the optimal medium conditions employed in that experiment. In most cases these optimum conditions were obtained when glucose was incorporated into the test medium.

The chromatography technique utilized for the detection of the presence of reducing sugars is described by Feigl (1954). The ascending method was elected, and paper cylinders were subjected to three or four eight-hour passages in water-saturated butanol at room temperature. Spots were developed by passing the paper through the following three solutions: 1) silver nitrate-saturated acetate, 2) 0.4 percent sodium hydroxide in methanol, and 3) ten percent sodium thiosulfate. Whatman No. 1 chromatography paper was routinely used. For extractions, Whatman No. 17 or Whatman 3 MM was used, in which case the test solution was put

on the filter paper in a line the length of the paper (rather than merely a spot). After three passages through the solvent, a one-half inch vertical paper strip was developed. From the location of bands on the strip, bands were marked across the paper, cut out, and extracted in a measured amount of distilled water by soaking the paper bands in water at 100°C for 30 minutes. Concurrent extractions of non-treated solutions attested to the efficacy of this extraction procedure.

RESULTS

Two inositolless isolates from the cross of _inos_ _a_ x _nic-1_ _al-2_ _A_ were selected to be used throughout this investigation. One of these isolates, _inos-8_, carries a mutant gene whose phenotype is characterized by its inability to utilize sucrose-sorbose-agar medium. The other isolate, _inos-9_, is characteristic of strains not carrying the mutant and possessing the normal faculty for utilization of sucrose-sorbose-agar medium. As such, future reference to _inos-8_ and to _inos-9_ will assume reference to the mutant form and to the normal form respectively.

Sheer trial-and-error experimentation led to perhaps the most important clue to _inos-8_'s inability to grow on sucrose-sorbose-agar medium. When agar was autoclaved separately from the salts-sucrose-sorbose portion of the medium, the viability of _inos-8_ conidia was greatly increased, as shown in Table 1. Note that the colony-forming efficiency of _inos-9_ was somewhat reduced by Westergaard's medium in which all components were autoclaved

together. This inos-9 response to sucrose-sorbose-agar medium

Table 1. Number of conidia of inos-8 and inos-9 forming colonies on medium in which agar was autoclaved separately from other components of the medium.

Method of media preparation	Number of conidia forming colonies*	
	inos-8	inos-9
(salts + sorbose + sucrose + agar)	0	146
(salts + sorbose + sucrose) + (agar)	165	200

*Average of three plate counts for inos-8 and two for inos-9.

has resulted in recovery ranging from 47 to 97 percent in different experiments, with the usual efficiency ranging from 80 to 90 percent. The more important increased colony-forming efficiency of inos-8 on medium in which the agar was autoclaved separately led to further juggling of components of the medium in an attempt to more closely characterize the inhibitory effect.

The Inhibitory Effect

The inhibitory effect is one compounded of three principal components of routine plating medium; sorbose, sucrose, and agar. These components can be varied individually, and their separate effects ascertained, but in the final analysis all must be present at the prescribed levels, and all must be autoclaved together to effect inhibition. The individual effects, insofar as it is possible to segregate them from the whole, are noted below.

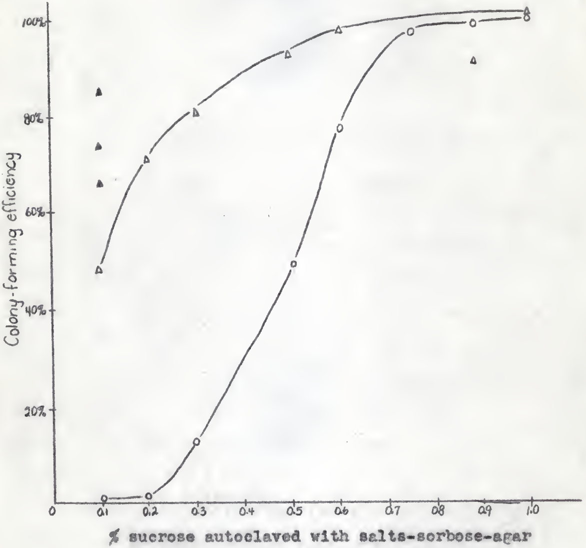
The Sucrose Effect. When the amount of sucrose in the sucrose-sorbose-agar plating medium was varied from 0.1 percent to one percent, the inhibitory effect on inos-8 was removed, even though all components of the medium were autoclaved together.

Employing a constant two percent agar and one percent sorbose level, the colony-forming response of <u>inos-8</u> and <u>inos-9</u> to increasing sucrose levels is summarized in Fig. 1. The increased viability of <u>inos-9</u> is analogous to data presented by de Serres, et al. (1962), in which a constant 0.1 percent sucrose level was maintained, and increased viability of wild type 74<u>A</u> conidia was proportional to increased autoclaving time of the medium.

Chromatographic determinations of non-autoclaved and autoclaved sucrose in water, with appropriate glucose and fructose controls, showed a single sucrose spot in the former, and sucrose plus fructose and glucose in the latter. No indication of sucrose contamination or formation of additional products during autoclaving, other than glucose and fructose, was obtained.

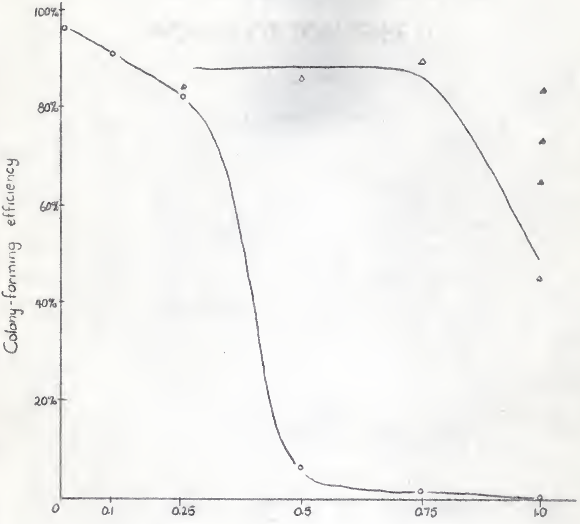
<u>The Sorbose Effect</u>. By decreasing the sorbose in sucrose-sorbose-agar medium from one percent to 0.25 percent, while maintaining a constant 0.1 percent sucrose and two percent agar level, the inhibitory effect on <u>inos-8</u> was again removed, as noted in Fig. 2. <u>inos-8</u> was highly sensitive to the normal sorbose level of one percent.

Non-autoclaved sorbose produced a single spot when it was chromatographed, indicating that there was no contamination by reducing sugars. Sorbose autoclaved in water, however, produced, in addition to the sorbose spot, a trail of four indistinct spots across the chromatogram, indicating that it was partially altered during autoclaving into substances which gave a positive reaction to the test for reducing sugars.



○ - inos-8
△ - inos-9
▲ - inos-9 data from other experiments. These points added to indicate that inos-9 efficiency on 0.1% sucrose-sorbose-agar medium in this experiment was the lowest ever obtained.

Fig. 1. Colony-forming efficiency of inos-8 and inos-9 on Westergaard's medium with constant 1% sorbose, 2% agar, and varying amounts of sucrose.



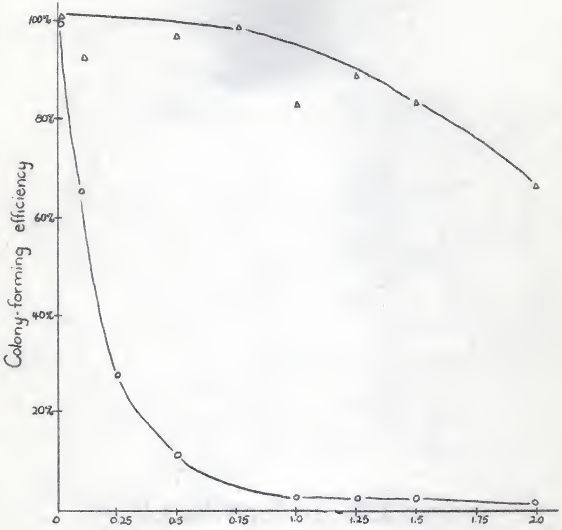
% sorbose autoclaved with salts-sucrose-agar

○ - inos-8

△ - inos-9

▲ - inos-9 data from other experiments. These points added to indicate that inos-9 efficiency on 1% sorbose-sucrose-agar medium in this experiment was the lowest ever obtained.

Fig. 2. Colony-forming efficiency of inos-8 and inos-9 on Westergaard's medium with constant 0.1% sucrose, 2% agar, and varying amounts of sorbose.



% agar autoclaved with salts-sucrose-sorbose

o - inos-8
Δ - inos-9

Fig. 3. Colony-forming efficiency of inos-8 and inos-9 on Westergaard's medium with constant 1% sorbose and 0.1% sucrose autoclaved with varying amounts of agar.

<u>The Agar Effect</u>. When the agar level was decreased to practically zero, <u>inos-8</u> was capable of increased colony-forming efficiency, as indicated in Fig. 3. Notice that <u>inos-9</u> was also somewhat inhibited by two percent agar. The obvious technical difficulty of too little agar for solidification was overcome by autoclaving the necessary amount of agar to bring the total level to two percent separately and double-strength in water. This was combined, after autoclaving, with double-strength sucrose-sorbose-agar, bringing the final concentration of agar autoclaved with the sugars and salts to that indicated in Fig. 3.

The results of chromatographing agar will be noted later.

Agar

<u>Dialysis and Chromatography</u>. It has previously been shown (Fig. 3) that <u>inos-8</u> is extremely sensitive to even low concentrations of agar autoclaved with sucrose and sorbose. The question arose as to whether any inhibitory agent could be removed from the agar. In an attempt to obtain a more purified agar Bacto-Agar washed up to 50 times, Difco Purified Agar, and Ionagar (Consolidated Laboratories, Inc.) were used as agar sources in the medium. None of these was capable of appreciably relieving the inhibitory effect, although thoroughly washed Bacto-Agar increased recovery of <u>inos-8</u> conidia from zero to ten percent. This latter information led to an investigation of the water-soluble portion of Bacto-Agar. The fact that washed agar had less inhibitory effect suggested that either some soluble

contaminant of agar or some component of agar itself was the inhibitory substance.

Agar is known to be a sulfuric acid ester of a linear polygalactose with a ratio of nine D-galactose molecules to one L-galactose molecule (Jones and Peat, 1942). Complete hydrolysis of agar with strong acid has yielded 40 percent D-galactose (because of excessive breakdown to levulinic acid), and methylation of agar has indicated about 11 percent L-galactose among the methanolysis products (Whistler, 1953). Yashe (1957) found that acid hydrolysis of agar resulted in the production of galactose, xylose, and hydroxymethyl-2-furaldehyde. The fact that agar is a polygalactose suggested that in its natural state agar might consist of a heterogeneous collection of chains of different lengths, and that some of these might be small enough to be soluble and to have lost their gel-forming properties.

Supernatent from the first washing of Bacto-Agar, when incorporated into the medium as (supernatent + salts + sorbose + sucrose) + (agar + water), resulted in the same inhibition as when agar per se was autoclaved with the salts and sugars. Agar was then dialyzed against a known volume of water, and the 24-hour dialysate was incorporated into the medium in the same manner as the supernatent. This too proved inhibitory, the colony-forming efficiency of inos-8 varying from 12 percent to 27 percent in different experiments with different dialysates. In order to determine whether the inhibitory capacity of the dialysate could easily be diluted out, dilutions were made of the dialysate and incorporated into the media as above. A normal

strength (1X) dialysate was made by using undiluted dialysate obtained from dialyzing ten g. agar against 250 ml. water. The results are summarized in Table 2. Data indicate that the

Table 2. Number of conidia of inos-8 and inos-9 forming colonies on Westergaard's media into which various dilutions of agar dialysate have been incorporated.

Dilution of dialysate incorporated as (dialysate + salts + sucrose + sorbose) + (agar + water)	No. of conidia forming colonies* inos-8	inos-9
2X	24	199
1X	34	175
1:5	173	205
1:25	216	201
1:50	279	180
1:100	238	197
1:200	236	195
1:400	227	---
1:800	213	213
distilled water control (no dialysate)	218	192
(agar + salts + sucrose + sorbose) control	15	194

*Average of three plate counts for inos-8, two for inos-9.

dialysate may be diluted five-fold and still retain certain inhibitory abilities. It is of interest that very dilute dialysate solutions (1:50) appear to be stimulatory to inos-8, for reasons not understood.

Separation of components of the agar dialysate by paper chromatography revealed the presence of two distinctly different components. Chromatography also indicated the presence of a non-reactive substance(s), appearing as a brilliant white band directly above the point of origin, in the dialysate. Extraction of these bands and of the line of origin from the paper and incorporation of these extracts into media gave the results shown in Table 3. It is obvious that the non-reactive band exerts the

most inhibitory effect. Certain of the other data may be explained by a consideration of the relative positions of the

Table 3. Colony-forming efficiency of inos-8 and inos-9 on Westergaard's media into which various components of agar dialysate isolated by paper chromatography have been incorporated.

Portion of dialysate autoclaved with salts, sorbose, and sucrose (agar autoclaved separately)	Colony-forming efficiency	
	inos-8	inos-9
untreated dialysate	0.18	0.95
non-reactive band	0.29	0.95
Band #1*	0.93	1.00
Band #2*	0.91	1.00
line of origin	0.58	0.94
Band #1 + Band #2	0.55	1.00
distilled water control (no dialysate)	1.00	0.96
(sucrose + salts + sorbose + agar) control	0.01	0.85

*These determinations made in a separate experiment.

isolated components. The developed strip appears as in Fig. 4. It is entirely possible that a small portion of the non-reactive band may have extended into the line of origin and into the region of Band #1 and been included in the extracted strips for both the line of origin and Band #1. This would more simply explain the

Fig. 4. Relative chromatogram positions of agar dialysate components.

1 2 3 4

(1) line of origin, (2) non-reactive band, (3) Band #1, (4) Band #2

inhibition evidenced by the line of origin and the combination of Band #1 and Band #2, although it is not inconceivable that substances from Band #1 and Band #2 may interact to cause inhibition.

The above results indicated the necessity for further investigation of the non-reactive portion of the dialysate, and experiments are now in progress in an attempt to identify this segment. Despite the fact that this soluble portion is readily dialyzed from whole agar, it must be recalled that agar treated in such fashion as to remove inhibitory levels of the substance still remains highly efficient in producing inhibition when autoclaved with salts and sugars. Agar of the highest commercial purity, as well as thoroughly washed agar, produced nearly 100 percent inhibition. Therefore the substance (or substances) responsible for inhibition appears to be integral to the agar structure, even though certain fractions are water-soluble and dialyzable. The continued presence of a brilliant white band during development of a chromatogram may indicate the presence of an acid substance which neutralizes the basic developing solution, or the presence of salts may show this defined non-reactivity. An inquiry to Difco Laboratories concerning possible impurities in Difco Bacto-Agar revealed that small amounts of chloride and sulfate were often present. However, the proportions of these were so low as to be insignificant in comparison to the chloride and sulfate levels present in the basal salts of Westergaard's medium.

It is interesting to speculate that the presence in the dialysate of a sulfated galactose fraction might be non-reactive to silver nitrate. When an extract of this non-reactive band was evaporated to dryness and heated in a crucible for 15 minutes

over a direct flame, excessive charring occurred, attesting to the organic nature of the extract. Although attention is directed to a particular portion of the agar, within this range of focus a panoramic view must be maintained which includes the added necessity of interaction with sucrose and sorbose in producing inhibition on one particular mutant type.

Conditions Which Simulate the Agar Effect. Heparin. In the study of viral inhibition caused by agar media (Takemota and Liebhaber, 1961), the inhibitory agent was identified as a sulfated agar polysaccharide. Agar inhibition was simulated by the substitution of agar by heparin in the media, heparin also being a sulfated polysaccharide. In the present study, the incorporation of heparin (Heparin sulfate, Eli Lilly and Co.) into Westergaard's media also effected inhibition, although ten-fold heparin concentrations, over that reported necessary for viral inhibition, were required to cause significant inhibition in Neurospora. The results of the heparin studies are indicated in Table 4. As the data show, it was only when heparin was autoclaved with the salts, sorbose, and sucrose that it had any inhibitory action. Autoclaving of inhibitory levels of heparin separately from the salts and sugars relieved the inhibition on inos-8, just as was the case with agar. This of course suggests that it is the sulfated polysaccharide nature of agar itself which is responsible for inhibition. inos-9 was not affected by high levels of heparin. Attention should also be called to the stimulatory effect on both inos-8 and inos-9 of low heparin

concentrations.

Table 4. Colony-forming efficiency of inos-8 and inos-9 on media to which varying amounts of heparin have been added.

Amount of heparin autoclaved with Westergaard's salts, sorbose, and sucrose (agar autoclaved separately)	Colony-forming efficiency	
	inos-8	inos-9
none	0.88	0.72
0.001 mg./ml.	0.91	1.00
0.005 mg./ml.	1.00	0.81
0.01 mg./ml.	0.82	0.78
0.05 mg./ml.	0.71	0.78
0.10 mg./ml.	0.75	0.84
0.50 mg./ml.	0.41	0.80
1.00 mg./ml.*	0.22	0.97
1.50 mg./ml.*	0.16	0.97
(sucrose + sorbose + salts + agar) control	0.01	0.74
(sucrose + sorbose + salts) + (agar + 1.00 mg. heparin/ml.)*	1.00	1.00

*These determinations made in a separate experiment.

Fries' and Vogel's media. In a routine attempt to extend these findings and to compare these results with those of de Serres, et al. (1962), Fries' and Vogel's media were also tested to determine their relationship to the inhibitory effect previously defined with Westergaard's medium. Vogel's and Fries' sucrose-sorbose-agar media were inhibitory to inos-8 and relatively non-inhibitory to inos-9, as was Westergaard's. There was, however, one unexpected but very important difference. The inos-8 inhibition elicited by Fries' and Vogel's media was not relieved by autoclaving the agar separately from the salts-sucrose-sorbose portion of the media. Therefore, the "agar effect" was in evidence in the complete absence of agar or any agar derivative. Obviously some component of the basal salts of Fries' and Vogel's media exerted an effect comparable to that characterized by agar.

The differences among the three media are summarized in Table 5. The principal difference between Westergaard's and both Fries' and Vogel's media is the presence of ammonium ions in Fries' and Vogel's (as ammonium nitrate in both, and ammonium tartrate in Fries'), and their obvious absence in Westergaard's. The effect of adding ammonium nitrate to Westergaard's is summarized in Table 6. Neither the addition of ammonium nitrate to Westergaard's salts nor the substitution of potassium nitrate by ammonium

Table 5. Comparison of components of Fries', Vogel's, and Westergaard's media.

Chemical components of medium	Westergaard's	Fries'	Vogel's
$CaCl_2$, $MgSO_4$, KH_2PO_4, trace elements, biotin	+	+	+
NaCl	+	+	-
KNO_3	+	-	-
NH_4NO_3	-	+	+
$Na_3C_6H_5O_7 2H_2O$	-	-	+
$(NH_4)_2C_4H_4O_6$	-	+	-

Table 6. Number of conidia of *inos-8* and *inos-9* forming colonies on Westergaard's medium to which varying amounts of ammonium nitrate have been added.

Percent of NH_4NO_3 autoclaved with Westergaard's salts, sucrose, and sorbose (agar autoclaved separately)	Number of conidia forming colonies*	
	inos-8	*inos-9*
none	522	417
1.0%	820	371
2.0%	858	383
3.0%	896	401
4.0%	895	374

*Average of three plate counts for *inos-8*, two for *inos-9*.

nitrate resulted in increased inhibition when agar was autoclaved separately from the salts and sugars and ammonium nitrate. As a matter of fact, the higher concentrations of ammonium nitrate in

Westergaard's were actually stimulatory to inos-8. The explanation of the unexpected stimulation is unknown. These results are pertinent in that they vindicate ammonium ions from involvement in the inhibitory effect peculiar to Fries' and Vogel's media.

The second difference among the three media is the additional presence of ammonium tartrate in Fries' medium and of sodium citrate in Vogel's medium. When these components (citrate and tartrate) were autoclaved with agar separately from the sucrose, sorbose, and remaining-salts (Fries' basal salts minus ammonium tartrate) solution, results as indicated in Table 7 were obtained.

Table 7. Colony-forming efficiency of inos-8 and inos-9 when different methods of incorporation of citrate and tartrate into the media were used.

Method of media preparation	Colony-forming efficiency	
	inos-8	inos-9
Fries'		
(sucrose + sorbose + salts + tartrate) + (agar + water)	0.032	0.921
(sucrose + sorbose + salts + tartrate + agar)	0.026	0.958
(sucrose + sorbose + salts) + (tartrate + agar + water)	0.65	0.750
Vogel's		
(sucrose + sorbose + salts + citrate) + (agar + water)	0.078	0.97
(sucrose + sorbose + salts + citrate + agar)	0.118	0.87
(sucrose + sorbose + salts) + (citrate + agar + water)	1.00	1.00

These data show that citrate and tartrate, when autoclaved with salts-sorbose-sucrose solution, produced the same inhibitory effect so characteristic of agar. This explains the ineffectiveness of autoclaving agar separately when Fries' and Vogel's media were utilized. Citrate and tartrate are alike in their possession

of carboxyl end-groups. If these results are in any way due to the presence of carboxyl end-groups, one might expect similar results from other compounds containing carboxyl end-groups. In order to check this, sodium acetate and sodium succinate were incorporated into media in the same manner outlined in Table 7. The incorporation of these compounds into the media resulted in the same inhibition and recovery pattern indicated in Table 7 with citrate and tartrate. The absence of any such carboxyl end-groups in any known components of agar suggests that although these compounds simulate the general inhibitory effect of agar, their mechanism of action may be quite different.

Sucrose

Eventually one must return to the original premise that agar must be autoclaved with one percent sorbose and 0.1 percent sucrose to effect inhibition. The ominous agar effect can be completely obliterated by increasing the sucrose level or by substituting sucrose with only 20 percent as much glucose. Certainly a closer examination of the sucrose role is warranted.

Sucrose is a disaccharide which yields equal amounts of D-glucose and D-fructose when hydrolyzed by acids or by enzymes. The usual pH of Westergaard's basal medium is 5.0. Pigman (1957), p. 592, states that the optimum pH for enzymatic sucrose hydrolysis is probably 5.0 to 5.5. He also states, p. 520, that

> The ΔF^{o} for the hydrolysis of sucrose is of the order of -6600 calories per mole; this means that the reaction has a strong tendency to favor hydrolysis. This tendency is greatly reinforced by the high concentration of one of the

reactants, water, in an aqueous environment. The combination of these factors is responsible for the practically irreversible nature of the hydrolysis of sucrose.

The Influence of Sucrose Hydrolytic Products on the Inhibitory Effect. Westergaard's sorbose-salts-agar medium supplemented with 0.1 percent filter-sterilized sucrose (Millipore Filter, size 0.45μ) cannot support growth of either inos-8 or inos-9. While inos-9 could not grow on filter-sterilized sucrose-sorbose-agar medium, it responded very well to the same medium autoclaved for 15 minutes. The obvious explanation for this is that the hydrolysis of sucrose during autoclaving in an acid medium resulted in the release of glucose and fructose into the medium, and this glucose was capable of supporting inos-9 colony formation. Although inos-8 can readily utilize glucose, it was incapable of utilizing the same autoclaved sucrose-sorbose-agar medium which so readily supported the growth of inos-9. Therefore one might suppose that some additional sucrose hydrolysis product other than glucose, when autoclaved with sorbose and agar, competes with the glucose or in some way negates inos-8's ability to respond normally to the glucose from hydrolyzed sucrose. The obvious point of attack for investigation of this hypothesis is a determination of the effect of fructose upon inos-8's utilization of glucose, fructose and glucose being produced in equal quantities when sucrose is hydrolyzed. Table 8 illustrates the effect of adding fructose and sucrose to media containing glucose. The data indicate that fructose as such was

non-utilizable by the strains tested. It appears that neither fructose nor any other breakdown product from the hydrolysis of

Table 8. Number of conidia of inos-8, inos-9, and wild type 74a forming colonies when equal aliquots were plated on media with various sources of sugar.

Carbohydrate source autoclaved with Westergaard's salts, 1% sorbose, and 2% agar	Number of conidia forming colonies*		
	inos-8	inos-9	74a
0.05% glucose	113	73	244
0.1% sucrose	0	61	110
0.05% fructose	0	0	0
0.05% fructose + 0.05% glucose	121	86	221
0.05% glucose + 0.1% sucrose	124	78	219

*Average of three plate counts for inos-8, two for inos-9, and two for 74a.

sucrose is capable of diminishing inos-8's ability to utilize glucose.

inos-8 can, however, utilize 0.1 percent sucrose autoclaved separately from agar. It is of interest whether this sucrose, hydrolyzed by autoclaving and quite utilizable by inos-8, would be capable of returning to a non-utilizable or inhibitory state by subsequent subjection to autoclaving with agar. 0.1 percent sucrose was autoclaved with Westergaard's salts and sorbose for 15 minutes at 18 lb. pressure, then two percent agar was added, and the medium autoclaved again for 15 minutes at 18 lb. pressure. The colony-forming efficiency of inos-8 conidia on the resulting medium was not decreased below the efficiency on the control medium with salts-sucrose-sorbose solution autoclaved separately from agar and combined after autoclaving. It appears that once sucrose has been hydrolyzed in the absence of agar, further autoclaving in the presence of agar is incapable of returning it to

a non-utilizable condition. If the presence of agar and sorbose during sucrose hydrolysis some way alters the sucrose hydrolytic products so they are inaccessible to inos-8, the foregoing results are easily explainable. If the results are to be interpreted in terms of the formation of an inhibitory substance during the second autoclaving period, then this product appears totally ineffective in the presence of sucrose hydrolysis products formed during the initial autoclaving process. One might question whether the increased autoclaving time of the sucrose (30 minutes total) might not be an influential factor. Such is not the case. Sucrose-sorbose-agar medium autoclaved for 30 minutes was ineffective in increasing inos-8 colony-forming efficiency over that obtained with the same medium autoclaved for 15 minutes.

The sucrose aspect of inhibition is not diminuitively influenced by sorbose. One might suppose that medium with two percent agar and 0.1 percent sucrose, but lacking sorbose, would still be incapable of supporting inos-8 colony-formation. Inositolless mutants present a rather unique faculty for validation of this hypothesis (Beadle, 1944). By decreasing the inositol in the medium from five μg. per ml. to 0.25 μg. per ml., colonization of inositolless mutants can be induced in the absence of sorbose. On such a medium, containing Westergaard's salts, 0.25 μg. inositol per ml., 0.1 percent sucrose and two percent agar, inos-8 conidia are capable of 100 percent colony-forming efficiency. And once again the impossibility of selectively attributing inhibition to one particular substance becomes apparent. Attention

will now be directed to the actual sucrose hydrolysis process, with agar and without agar, with sorbose and without sorbose. Two different methods have been utilized in determining the influence of different components of the medium on sucrose hydrolysis; one was a biological study, the other a chromatographic approach.

Effects of Sorbose, Salts, and Agar on Sucrose Hydrolysis. Sucrose can be autoclaved with individual components of the medium and the effectiveness of sucrose hydrolysis determined by the response of the normal strain, *inos-9*, to the resulting medium. The concurrent effect on *inos-8* of the products utilizable by *inos-9* can be ascertained. The results of such an experiment are summarized in Table 9. Both Westergaard's salts and sorbose appear instrumental in the hydrolysis of sucrose. It has been postulated by de Serres, et al. (1962) that sorbose may catalyze sucrose hydrolysis. Such a postulate is strengthened by these data. Table 9 suggests that sucrose in the presence of sorbose may be hydrolyzed sufficiently to permit normal *inos-9* colony-forming efficiency, but insufficiently to obtain optimum recovery of *inos-8* (No. 3). Westergaard's salts were apparently capable of effecting hydrolysis of sucrose to the optimum recovery of both *inos-8* and *inos-9* when agar and sorbose were autoclaved separately (No. 7), but for reasons which are not clear the *inos-8* recovery was diminished when sorbose and agar were autoclaved together (No. 4), even though sucrose was autoclaved only with salts; there was an added agar-sorbose effect associated with the

inhibitory effect on inos-8. Notice that neither inos-8 nor inos-9 was capable of utilizing sucrose autoclaved by itself or

Table 9. Number of conidia of inos-8 and inos-9 forming colonies on media prepared in such a manner as to permit an assay of the degree of sucrose hydrolysis.

Method of media preparation (Westergaard's salts, 0.1% sucrose, 1% sorbose, 2% agar)	Number of conidia forming colonies*	
	inos-8	inos-9
1. (sucrose + sorbose + salts + agar)	0	166
2. (sucrose + sorbose + salts) + (agar)	216	304
3. (sucrose + sorbose) + (salts + agar)	162	307
4. (sucrose + salts) + (sorbose + agar)	45	314
5. (sucrose) + (sorbose) + (salts + agar)	0	9
6. (sucrose) + (sorbose) + (salts) + (agar)	0	11
7. (sucrose + salts) + (sorbose) + (agar)	248	312
8. (sucrose + agar) + (sorbose + salts)	0	0

*Average of three plate counts for inos-8, two for inos-9.

sucrose autoclaved only with agar (Nos. 5, 6, and 8). It may or may not be significant that the response of inos-9 to sucrose autoclaved only with agar was zero. The fact that two different experiments incorporating this same variable have failed to give any colonies with inos-9 or wild type 74a conidia lends credance to the supposition that it is significant. If it can be assumed that sucrose in the presence of agar is hydrolyzed to a lesser degree than sucrose autoclaved in distilled water, then variable No. 1, the basis of the inhibitory effect, in Table 9 might be explained in terms of this assumption. The arguments for and against such an interpretation will be more thoroughly developed

in the discussion.

Chromatographic determinations of the extent of sucrose hydrolysis when sucrose was autoclaved with various components of the medium have been on the whole quite unsatisfactory with the one-dimensional technique. The technical difficulty encountered was that the sorbose spot was located directly over the fructose and glucose regions, and since sorbose was present in a ten-fold concentration of the sucrose level, there was never any discernible indication of the degree of glucose and fructose formation, due to a heavy saturation of the region by sorbose. This difficulty could possibly be resolved by utilizing different chromatographic techniques. There was some indication that sucrose autoclaved with agar was not hydrolyzed to the same extent as was sucrose autoclaved with water (no sorbose). The glucose spot was not discernible in the former, but there was slight indication of glucose formation in the latter. There was definite indication, once again in terms of the density of the resulting glucose spot, that one percent sucrose autoclaved in water was hydrolyzed to a much greater degree than was 0.1 percent sucrose autoclaved in water, even though the latter was concentrated ten-fold by evaporation at 60°C before spotting. When all components of the media were autoclaved together, chromatograms indicated the presence of an unidentified and reproducible substance locating distal to the sorbose region, and present only when all components were autoclaved together. Further investigation of this substance is being undertaken.

Could the presentation of results cease at this point, interpretation would be greatly simplified. Results have indicated that sucrose hydrolysis products are either inaccessible, or are available in quantities much too low to support inos-8 colony formation. However, contradictory data cannot be ignored.

Inhibitory Substance

inos-8 conidia grown in liquid medium containing sorbose and sucrose (no agar) were capable of forming visible colonies after two to four days' incubation, as would be expected. The number of inos-8 conidia forming colonies appeared roughly equal in sucrose-sorbose and glucose-sorbose liquid media. The question arose as to whether inos-8 conidia which had germinated and begun to grow in liquid would be able to continue growth to form a visible colony when transferred to sucrose-sorbose-agar medium. Microscopic determinations of the conidial germination of inos-8 on sucrose-sorbose media indicated that after four hours, approximately 90 percent of the conidia had germinated.

Flasks with 150 ml. of Westergaard's sucrose-sorbose medium and others with 150 ml. glucose-sorbose medium were inoculated with inos-8 and inos-9 conidial suspensions resulting in a final concentration of about 1,000 conidia per ml. of media (0.1 percent agar was added to the media to produce a slight viscosity which prevented germinating conidia from aggregating). At intervals of time from one hour to 24 hours, one ml. of conidial suspension was removed, diluted 1:10, and plated on three plates of

sucrose-sorbose-agar medium and three plates of glucose-sorbose-agar medium. The colony-forming efficiency was determined by a comparison of the sucrose-sorbose-agar counts to the corresponding glucose-sorbose-agar counts (glucose-sorbose-agar counts considered 100 percent). The results of these determinations are shown in Fig. 5.

inos-8 conidia were capable of growing normally in sucrose-sorbose as well as glucose-sorbose liquid media in shake culture. However, a certain incubation time had to elapse before conidia growing in such liquid media could withstand the transfer to sucrose-sorbose-agar medium and maintain sufficient equilibrium to continue growth and form a colony. Following a lag period, increase in inos-8 colony-forming efficiency was found to be proportional to the amount of time the conidia had incubated in liquid media. Even inos-9 did not immediately show normal colony-forming efficiency. The lag period of inos-8, however, exceeded the time required by inos-9 to reach full colony-forming efficiency. It was relatively unimportant to final recovery whether inos-8 conidia were incubated in sucrose or glucose liquid, although response was somewhat more rapid when glucose was used. It appears that a conidium must reach a certain physiological state, logically a certain physical size, for it to be capable of overcoming the adverse affects of sucrose-sorbose-agar medium on which it is plated and continue development on such a medium to form a colony. This suggests the possibility of a sucrose-sorbose-agar threshold inhibitory level; the greater the fungal

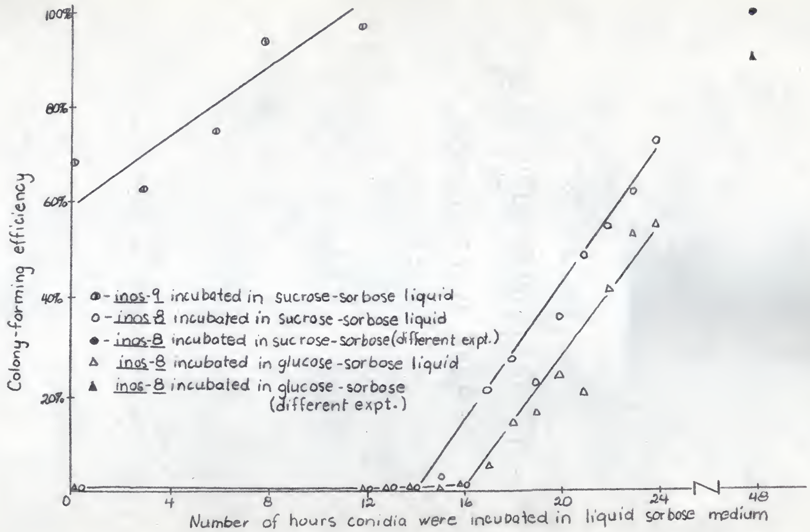


Fig. 5. Colony-forming efficiency of _inos-8_ and _inos-9_ on sucrose-sorbose-agar medium following incubation for various intervals of time in Westergaard's liquid medium supplemented with sorbose and glucose or sucrose.

surface area per unit of "inhibitor", the greater the possibility of a breakthrough, due to the adsorption or neutralization of the inhibitor substances by the increased mycelial area, leaving other growing sites unaffected and free to continue growth. This hypothesis predicts that the greater the surface area subjected to a constant amount of inhibitor, the greater is the possibility of survival, or, equally, the smaller is the probability of total inhibition. It also presupposes that sufficient carbon sources are available in the sucrose-sorbose-agar medium to support <u>inos-8</u> growth, but utilization of these sources is impaired by the presence of a separate inhibitory substance, toxic to <u>inos-8</u>, but hardly affecting <u>inos-9</u>.

The postulate that the inhibitory substances are present in finite numbers can be tested further by plating increasing numbers of <u>inos-8</u> conidia on sucrose-sorbose-agar medium with corresponding platings on glucose-sorbose-agar control plates. Theoretically, an inhibitory substance saturation level might be attained, above which <u>inos-8</u> conidia would be capable of colony formation on sucrose-sorbose-agar medium. The result of plating conidial samples of increasing concentrations on these media indicated once more that there was a level above which the inhibitory effect was markedly decreased. An <u>inos-8</u> conidial suspension giving rise to an average of 415 colonies on glucose-sorbose-agar medium produced one colony on sucrose-sorbose-agar (average of three plate counts each), or a colony-forming efficiency of 0.0024 on sucrose-sorbose-agar medium. When approximately 8,000

inos-8 conidial colonies were formed on glucose-sorbose-agar plates, 1,264 conidia formed colonies on sucrose-sorbose-agar medium, an efficiency of approximately 0.16. Obviously, these latter determinations are only approximate, but it is of basic importance in terms of interpretation to realize that inos-8 conidia are fully capable, under the conditions imposed by plating very large numbers of conidia, of forming normal colonies on sucrose-sorbose-agar medium. Could the conidial concentrations be increased even more, it does not seem impossible that inos-8 sucrose-sorbose-agar colony-forming efficiency could equal the glucose-aorbose-agar efficiency. This becomes operationally difficult and impossible, because colony counts above 500 are increasingly unreliable, scoring becomes very laborious, and results are approximations at best.

Characteristics and Inheritance Studies

The inos-8 strain is typical of a mutant type characterized by its inability to form colonies when conidia are plated on sucrose-sorbose-agar medium. The mutant characteristic can be transferred through a cross to other nutritionally-deficient strains, and to strains lacking nutritional requirements (wild types). When this mutant is associated with inositol-requirement, it has been found to be morphologically identifiable by its production of dark brown pigmentation which accumulates in the medium. The mutant characteristic is very stable, with no bona fide spontaneous back-mutations encountered throughout the entire

study. Occasionally a low frequency of colonies was found when strains of the mutant type were plated on sucrose-sorbose-agar medium. Subsequent culturing of these colonies and re-plating of conidia on sucrose-sorbose-agar medium with glucose-sorbose-agar controls resulted in the usual mutant colony-forming efficiency of essentially zero.

In order to determine the segregation pattern of this mutant in a cross, inos-8 was crossed to a pan-1 al-1 A strain possessing the normal ability for utilization of sucrose-sorbose-agar medium. Isolation and testing of random ascospores from this cross indicated the segregation for normal and mutant types summarized in Table 10. When data from all four types were pooled,

Table 10. Segregation of mutant and normal types in a cross of inos-8 x pan-1 al-1 A.

Number of isolates tested	Nutritional requirement	Proportion of mutant types	Proportion of normal types
52	pan, inos	0.442	0.557
64	pan	0.265	0.734
27	inos	0.741	0.259
39	none	0.538	0.461
172 total		0.498*	0.501*

*Average proportion.

there was a nearly perfect 1:1 segregation for mutant and normal isolates among the progeny. Within this pooled determination, however, there were two types which deviated significantly from the combined 1:1 ratio. The fact that these deviants showed a 1:3 and a 3:1 segregation for mutant and normal types made them especially intriguing from a genetic standpoint. Attempts have been made and experiments designed to explain the unusual segre-

gation in terms of linkage, epistasis, and hypostasis, all to no avail. Further crosses of isolates within the deviant categories to normal and mutant strains have failed thus far to lend any insight into the genetic basis of the mutant phenotype. An analysis of products of a single ascus, followed by an analysis of the progeny of crosses made with these single-ascus isolates would hopefully elucidate the genetic mechanism. At present, the investigator is forced to attribute the deviant segregants to a rather general interaction of the particular nutritional requirements of a strain with their association with the mutant type under study. It is possible that certain genotypes might react to one set of variables in a characteristic manner, and to another set of variables in a completely different manner. It would be quite informative to establish the reaction of these deviant mutant isolates to all the various conditions affecting inos-8.

Rather than pursue this same line of investigation, inos-8 was crossed to pan-2 ad-8 5A, a strain with genetic markers on a different chromosome from the pan-1 strain previously studied. In this instance the inheritance was straightforward. In an examination of 97 isolates from this cross, a good fit for a ratio of one normal to one mutant type (verified by the chi-square test, p values all above 0.05) was obtained regardless of the nutritional requirement of the isolate. The data from these determinations are summarized in Table 11. In the progeny of this cross the mutant behaves in every way as a one-gene characteristic.

Table 11. Segregation of mutant and normal types in a cross of inos-8 x pan-2 ad-8 5A.

Number of isolates tested	Nutritional requirement	Proportion of mutant types	Proportion of normal types
17	inos	0.471	0.529
18	none	0.555	0.444
26	pan and/or adenine*	0.615	0.384
36	pan, inos and/or adenine, inos	0.583	0.416
97 total		0.556***	0.443***

*Grew on media supplemented with pantothenate and adenine, but not on minimal or inositol-supplemented media.

**Grew on media supplemented with pantothenate, adenine, and inositol, but not on pantothenate-adenine-supplemented, inositol-supplemented, or minimal media.

***Average proportion.

CONCLUSIONS AND DISCUSSION

The inhibitory effect has been defined in terms of the inability of conidia of certain mutant strains to form colonies when plated on Westergaard's sucrose-sorbose-agar medium. It should be emphasized that the strain-8 identified with the inositolless marker is simply a carrier of the mutant under consideration. The mechanism of action responsible for the mutant phenotype of the strains of which inos-8 is typical is deserving of principal consideration.

It is hard to believe at present that the mutant's effects are so varied as to be separately affected by the wide variety of experimental conditions used in the present study. Rather, it would appear more likely that its impaired function is collectively affected by a variety of environmental conditions. It is apparent that the reduced ability of inos-8 conidia to utilize the

hydrolysis products of sucrose is a part of its phenotype, but this is only expressed under the restricted condition of growth imposed by the presence of sorbose in the medium. It is tempting to speculate that the mutant effect is in some way related to biological processes in which the cell wall or membrane plays a dominant role. de Terra and Tatum (1961) have shown that sorbose alters the cell wall constitution of Neurospora by decreasing the amount of glucose and increasing the amount of glucosamine in the cell wall over that present in the normal cell wall. Conversely, treatment of Neurospora with snail digestive enzymes known to structurally alter cell wall composition resulted in colonization of Neurospora indistinguishable from sorbose colonization. That the sorbose effect is a surface phenomenon is substantiated by the observation that aerial mycelia arising from sorbose-induced colonies possess the normal morphology of mycelia grown on non-sorbose media. Further evidence has been provided by Wilson (see de Terra and Tatum, 1961), who has shown that the micro-injection of sorbose at levels found in sorbose-induced colonies into Neurospora hyphae failed to restrict normal growth. Although these investigators have morphologically described the effect of sorbose on growth of Neurospora, the chemical mechanism of the sorbose action is still incompletely understood.

Since a substantial decrease in concentration of sorbose or its complete elimination from salts-sucrose-agar medium restores normal colony-forming efficiency to inos-8, it is apparent that the impaired function of inos-8 to grow on sucrose-sorbose-agar

medium is manifested only in the presence of sorbose. Consequently the mechanism of action of the mutant gene under consideration might well be inter-related to the biochemical alteration of the cell wall imposed by the presence of sorbose in the medium. This restriction of sugar utilization imposed by sorbose upon inos-8 is however completely relieved when glucose is added to the medium. It is only with sucrose that any inhibition is evidenced.

A careful examination of the various factors affecting inos-8 makes it clear that they cannot all be satisfactorily explained under a single hypothesis. The inhibitory effects of sucrose-sorbose-agar medium are of such a nature that they will tentatively be divided into two groups, a different hypothesis being used to provisionally explain each. It is imperative that the reader be fully cognizant of the distinction between the two. One submits that under certain conditions the sucrose hydrolysis products are unavailable to inos-8, either because the hydrolysis products of sucrose are present in a non-utilizable form, or because the degree of sucrose hydrolysis is insufficient to produce high enough levels of glucose to support inos-8 colony formation, the requirements of inos-8 being greater than those of inos-9 on sucrose-sorbose-agar medium. Perhaps the best direct evidence for this postulate is the complete relief of inos-8 inhibition when 0.02 percent glucose is added to the routine sucrose-sorbose-agar medium. The other interpretation presupposes the presence of sufficient levels of sugars in a utilizable form to support

inos-8 colony formation, but submits that a separate inhibitory product either prevents inos-8's utilization of these sucrose hydrolysis products or in some other manner hinders the normal metabolism of inos-8 on sucrose-sorbose-agar medium. The best support for this hypothesis is the marked increase in inos-8 colony formation on sucrose-sorbose-agar medium when such medium has been saturated with very large amounts of living cell wall surface area, either as conidial or as mycelial cells.

Further evidence supporting each of these two contradictory interpretations will be noted, as well as evidence which can be attributed to both. Certain data will be explained in terms of one hypothesis only because they do not fit the conditions required for inclusion in the other. Also, some of the data must be interpreted in terms of individual rather than general effects.

The factors affecting the colony-forming efficiency of inos-8 are many and varied, and all probably reflect the phenotypic nature of the mutant under consideration. Perhaps these factors can be placed in better perspective by first reviewing the conditions which affect the colony-forming efficiency of the normal strain, inos-9. There are only two conditions which can appreciably decrease inos-9's colony-forming efficiency on media containing 0.1 percent sucrose, one percent sorbose, two percent agar, and a basal salts solution. The most striking effect is caused by autoclaving sucrose by itself in solution or in solution in the presence of only agar. Apparently under such circumstances the degree of sucrose hydrolysis is so slight as to be

practically negligible, and even normal inos-9 and wild type 74a cannot utilize such media. The second condition affecting inos-9 is the general inhibitory effect of autoclaved Westergaards sucrose-sorbose-agar medium. This medium results in inos-9 colony-forming efficiency ranging from as low as 47 percent to as high as 97 percent, with the average efficiency being from 80 to 90 percent. This inhibition has been alleviated by extending the autoclaving time of the media from 15 to 30 minutes (present study, and de Serres, et al., 1962). This suggests that inhibition is due to an inadequate carbon source, in that extended autoclaving increases sucrose hydrolysis, producing larger amounts of glucose and relieving the inhibition. Such inhibition of normal strains can be attributed to inadequate carbon levels due to ineffective sucrose hydrolysis (in agreement with de Serres, et al., 1962).

On the other hand, the factors affecting inos-8 are much more numerous than those affecting inos-9 and not nearly as straightforward in terms of interpretation. Such factors are considered below.

1. The general inhibitory effect of Westergaard's 0.1 percent sucrose-one percent sorbose-two percent agar medium reduced inos-8's colony-forming efficiency to less than one percent (Table 1). This inhibitory effect may be attributed to either or both of the two hypotheses. All other experiments have been designed in an attempt to establish a distinction between the two interpretations based upon this original finding.

2. The colony-forming efficiency of inos-8 on sucrose-sorbose-agar medium can be raised to nearly 100 percent by increasing the sucrose level from the standard 0.1 percent to one percent (Fig. 1). This finding very strongly supports the non-availability theory, and suggests that by simply increasing the amount of sucrose available for hydrolysis, increased amounts of hydrolytic products become available and inos-8 is able to function normally.

3. Reduction of sorbose in sucrose-sorbose-agar medium from the normal one percent to 0.25 percent relieved inos-8 inhibition (Fig. 2). inos-8 was fully capable of utilizing Westergaard's medium containing 0.1 percent sucrose and two percent agar when limiting-inositol was used to restrict growth instead of sorbose. These results suggest that 0.1 percent sucrose provides sufficient hydrolytic products to efficiently support inos-8 colony formation in the absence of sorbose. Addition of one percent sorbose imposes new restrictions and inos-8 can no longer utilize 0.1 percent sucrose, which supports the hypothesis of the formation of an actual inhibitory substance. The addition of one percent sorbose may also supply the missing element in the sucrose-sorbose-agar triad necessary to cause alteration of the sucrose hydrolysis products so that they become inaccessible to inos-8.

4. Preparation of the medium with separate autoclaving of the agar resulted in inos-8 colony-forming efficiency approaching 100 percent (Table 1). This suggests rather strongly that there is a sucrose-sorbose-agar complex which is inhibitory to inos-8.

None of these substances is detrimental in itself, and the overall inhibition can be relieved by separate autoclaving of the agar. However, these results might also indicate that the hydrolysis products of sucrose autoclaved with sorbose and agar are merely altered in some slight manner so as to be non-utilizable by inos-8.

5. inos-8 has the same sensitivity (colony-forming efficiency greatly reduced) to agar dialysate or agar supernatent autoclaved with the salts-sucrose-sorbose separately from the agar as it does to whole agar autoclaved with salts-sorbose-sucrose (Tables 2 and 3). These results indicate that there is a soluble and dialyzable portion of the agar which is capable of causing the same inhibition as whole agar, or perhaps once agar is autoclaved, the same dialyzable inhibitory product is formed as a result of autoclaving.

6. One mg. of heparin per ml. of media autoclaved with salts-sucrose-sorbose separately from the agar is inhibitory to inos-8 (Table 4). inos-8 is not affected by the same media in which the salts-sorbose-sucrose is autoclaved separately from inhibitory levels of heparin plus agar. This suggests that it is the sulfated-polysaccharide nature of agar which is responsible for the role exerted by agar in the inhibitory effect.

7. inos-8 is sensitive under certain conditions to the sodium citrate and ammonium tartrate components of Vogel's and Fries' media as well as to sodium acetate and sodium succinate, even when these salts were autoclaved separately from the agar

(Table 7). Inhibition can be relieved by autoclaving these substances with the agar separately from salts-sucrose-sorbose portion of the media. These substances mimic the agar effect in every aspect, but their identification with the chamical structure of agar has not been established. These data suggest a possibly wider area of inhibition than has been so far investigated.

8. inos-8 cannot utilize media in which the sucrose was filter-sterilized, autoclaved in water, or autoclaved alone with agar in water (Table 9). This is in agreement with de Serres, et al. (1962) that sucrose must first be hydrolyzed to glucose and fructose before it can be utilized in sucrose-sorbose-agar media.

9. The colony-forming efficiency of inos-8 is nearly 100 percent when 0.05 percent glucose is substituted for 0.1 percent sucrose in Westergaard's glucose-sorbose-agar medium (Table 8). Such efficiency was unaltered by separate autoclaving of the agar. Neither the addition of fructose nor the addition of sucrose to glucose-sorbose-agar medium was instrumental in decreasing the response of inos-8 to glucose-sorbose-agar medium. These data strongly support the non-availability theory. Were there some inhibitory substance formed during sucrose-sorbose-agar autoclaving, one would expect that the colony-forming efficiency of inos-8 would be appreciably reduced on that medium, even when glucose was added. The fact that the addition of glucose completely relieved inos-8 inhibition on any otherwise-inhibitory

medium suggests that the inhibitory effect is due to the absence of sufficient sugars.

10. Autoclaving sucrose-sorbose-agar media for 30 minutes at 18 lb. pressure did not increase inos-8's colony-forming efficiency over that obtained with the same media autoclaved for 15 minutes at 18 lb. pressure (p. 26). This suggests that increased hydrolysis of sucrose is ineffective in relieving inhibition and indicates rather strongly the formation of an inhibitory substance. The possibility that even increased amounts of sucrose hydrolysis products if some way altered may be non-utilizable by inos-8 cannot be overlooked.

11. When salts-sucrose-sorbose medium was autoclaved for 15 minutes at 18 lb. pressure, followed by the addition of two percent agar, and a second autoclaving at 18 lb. pressure for 15 minutes, the inos-8 colony-forming efficiency was somewhat higher than it was on medium in which the salts-sucrose-sorbose portion of the medium had been autoclaved entirely separately from the agar (p. 25). These results are attributed to the non-availability of sugars, mainly because the results oppose those expected from an inhibitory substance. Once sucrose-sorbose has been autoclaved in the absence of agar and sufficient hydrolysis products released, further autoclaving in the presence of agar actually somewhat increases inos-8 colony-forming efficiency over that in which the salts-sucrose-sorbose portion of the medium had been autoclaved entirely separately from the agar.

12. Both Westergaard's salts and sorbose affected the hydro-

lysis of sucrose so that sucrose hydrolysis products became utilizable by inos-8 when agar was autoclaved separately (Table 9). There was an unexpected decrease in inos-8 colony-forming efficiency when agar was autoclaved with sorbose separately from sucrose and salts. The affect of sorbose upon sucrose hydrolysis might be explained by the fact that sorbose breaks down readily when autoclaved. These sorbose breakdown products could conceivably be acid in nature, and the acid environment could result in rather rapid sucrose hydrolysis. The acidic nature of Westergaard's salts solution is undoubtedly responsible for its ability to cause hydrolysis of sucrose. The sorbose-agar interaction remains unexplained.

13. inos-8 conidia incubated in sucrose-sorbose and glucose-sorbose liquid media for periods up to 48 hours became increasingly capable of continuing growth to form a colony when transferred to sucrose-sorbose-agar medium, the colony-forming efficiency being proportional to the incubation time in liquid media, following a 14-hour lag (Fig. 5). This is evidence in support of the inhibitory substance hypothesis. Once the surface area of a potential colony has been increased by conidial germination and repeated branching of the mycelia to a certain point, the inhibitory substance is no longer capable of affecting all the reactive sites to prevent further development, and the organism continues growth to form a colony. Sufficient utilizable sugars must be present in the sucrose-sorbose-agar medium to support continued metabolism and growth, although it is not entirely

impossible that germinating inos-8 conidia may have absorbed sufficient utilizable sugars from the liquid media to enable them to continue growth on an otherwise inhibitory sucrose-sorbose-agar medium. This possibility requires further study.

14. When the number of inos-8 conidia plated on sucrose-sorbose-agar plates was increased to the thousands, colony-forming efficiency was significantly increased in comparison to corresponding glucose-sorbose-agar counts (p. 34). This line of evidence needs better data, but those available implicitly imply the presence of an inhibitory substance with a finite saturation level, and the presence of sufficient utilizable carbon sources in the sucrose-sorbose-agar media to support colony formation.

As has been constantly stressed throughout this presentation, the inhibitory effect is one inseparably compounded of a sucrose-sorbose-agar interaction. However, certain components of the agar have been studied separately, and the effects of these upon the general inhibitory effect determined. Mutant strains of poliovirus (Takemori and Nomura, 1960) and EMC virus (Takemoto and Liebhaber, 1961) have been isolated which form morphologically aberrant plaques when overlayered with agar medium. The inhibitory portion of the agar has been partially purified and identified as a non-dialyzable sulfated polysaccharide by Takemoto and Liebhaber (1961). Heparin, a sulfated polysaccharide, and sulfated polyglucose were also found by these workers to be inhibitory to EMC mutant virus when incorporated into the media at the same concentration as agar polysaccharide. Heparin

in much higher concentrations was also inhibitory to inos-8, but not to inos-9. The principal difference between the Neurospora and virus inhibition is that the inhibitory agar portion affecting viruses is non-dialyzable, as would be predicted of a polysaccharide, while the inhibitory agar component affecting Neurospora mutants is dialyzable. Dialysis of agar, however, in no way decreased the inhibitory effect of that same dialyzed agar when it was autoclaved and incorporated into sucrose-sorbose-agar medium. Not to be ignored is the possibility of two different substances' in commercial agar being responsible for effecting inhibition of inos-8; one dialyzable and perhaps non-specific in that citrate, tartrate, acetate, succinate, and possibly an even wider range of compounds are capable of mimicking it, and the other non-dialyzable and an integral part of the agar structure, namely the sulfated agar polysaccharide described by the virus investigators whose effect is simulated by heparin.

An unexplained agar stimulation of normal poliovirus strains has been reported by Takemori and Nomura (1960). A comparable stimulation has been observed in this study with low concentrations of agar dialysate and heparin. The explanation of this stimulation remains obscure, but it is interesting to postulate that a given substance may be inhibitory at high concentrations and at the same time be beneficial and stimulatory in low concentration.

The inheritance of the mutant type was completely straightforward in the examination of 97 isolates from a cross of inos-8

x pan-2 ad-8 5A, the progeny segregating 1:1 for mutant and normal phenotype. In a cross of inos-8 x pan-1 al-1 A, although the pooled ratio of normal to abnormal progeny was 1:1, the inheritance within the four nutritionally-deficient types was compounded by other factors. It is possible that modifier genes or genes with epistatic action may also have been segregating. The fact that an inos-8 cross to a strain with markers on a separate chromosome (pan-2) produced progeny which segregated normally suggests that linkage may also have complicated the analysis.

The final decision as to whether the inos-8 inhibitory effect is a result of inaccessibility of the sucrose hydrolysis products or is in fact the reflection of the presence of an inhibitory substance must, unfortunately, await refined experimentation. To the investigator the balance at present is somewhat tipped in favor of the non-availability interpretation. There is, however, the not too remote possibility that both hypotheses may be correct, and that certain conditions merely produce effects which lend themselves more readily to interpretation by one hypothesis than the other. There are also many experiments which defy explanation by one interpretation to the exclusion of the other. Hopefully further experimentation will succeed in providing the answer.

ACKNOWLEDGMENT

The investigator wishes to express sincere gratitude to Dr. Thad Pittenger, her major instructor, for indispensable assistance and guidance throughout the course of the present study. She is also indebted to Dr. Philip Nordin for his interest and suggestions concerning the carbohydrate biochemistry of the problem, as well as his aid in making chromatographic determinations.

REFERENCES

Beadle, G. W.
An inositolless mutant strain of Neurospora and its use in bioassays. J. Biol. Chem. 156:683-689. 1944

Barratt, R., D. Newmeyer, D. D. Perkins, and L. Garnjobst.
Map construction in Neurospora crassa. Advances in Genet. 6: 1-93. 1954.

Case, M. E., and N. H. Giles.
Evidence from tetrad analysis for both normal and aberrant recombination between allelic mutants in Neurospora crassa. Proc. Natl. Acad. Sci. U.S. 44:378-390. 1958.

Clark, William Arthur.
Effect of carbohydrates on some Myxobacteria. J. Bacteriol. 67:589-592. 1954.

de Serres, F. J., H. G. Kolmark, and H. E. Brockman.
Factors influencing the survival of Neurospora crassa conidia in sorbose-sucrose media. Nature 193:556-557. 1962.

de Terra, Noel, and E. L. Tatum.
Colonial growth of Neurospora. Science 134:1066-1068. 1961.

Feigl, Fritz.
Spot tests: organic application. Vol. 2. New York: Elsevier Publishing Co., 1954.

Horowitz, N. H., and G. W. Beadle.
A microbiological method for the determination of choline by use of a mutant of Neurospora. J. Biol. Chem. 150:325-333. 1943.

Ishikawa, T.
Complementation and genetic maps of ad-8 locus in Neurospora crassa. Genetics 45:993. 1960.

Jones, W. G. M., and S. Peat.
The constitution of agar. J. Chem. Soc. 225. 1942.

O'Neille, A. N., and D. K. R. Stewart.
On the structure of agar from Gelidium cartilaginum. Can. J. Chem. 34:1700-1703. 1956.

Pigman, Ward.
The carbohydrates. New York: Academic Press, 1957.

Shatkin, A. J., and E. L. Tatum.
The relationship of m-inositol to morphology in Neurospora crassa. Am. J. Botany 48:760-771. 1961.

Takemori, N., and S. Nomura.
Mutation of polioviruses with respect to size of plaque. II. Virology 12:171-184. 1960.

Takemoto, K. K., and H. Liebhaber.
Virus-polysaccharide interactions. 1. An agar polysaccharide determining plaque morphology of EMC virus. Virology 14: 456-462. 1961.

Tatum, E. L., R. W. Barratt, and V. M. Cutter.
Chemical induction of colonial paramorphs in Neurospora and Syncephalastrum. Science 109:509. 1949.

Vogel, H.
A convenient growth medium for Neurospora. Micro. Genet. Bull. 13:42. 1956.

Westergaard, M., and H. K. Mitchell.
Neurospora V. A synthetic medium favoring sexual reproduction. Am. J. Botany 34:573-577. 1947.

Whistler, R. L., and C. L. Smart.
Polysaccharide chemistry. New York: Academic Press, 1953.

Yaphe, W.
The use of agarase from Pseudomonas atlantica in the identification of agar in marine algae (Rhodophyceae). Can. J. Microbiol. 3:987-993. 1957.

THE INHIBITORY EFFECT OF SUCROSE-SORBOSE-AGAR MEDIUM ON A MUTANT OF *NEUROSPORA CRASSA*

by

TRUDY BRAWNER

B. S., Kansas State University, 1959

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

GENETICS

Department of Agronomy

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1962

One of the most useful experimental techniques available to the Neurospora geneticist involves a method now routinely used which induces colonization of Neurospora mycelia. It is the supplementation of the regular synthetic medium with 0.1 percent sucrose and one percent sorbose which induces colony formation and thereby facilitates the use of bacteriological plating procedures in the study of fungi. Recently, a unique mutant strain has been isolated which is characterized by the failure of its conidia to germinate and form colonies on standard plating medium. Oddly enough, this inhibition of growth on routine medium can be relieved simply by the substitution of 0.02 percent glucose for the 0.1 percent sucrose.

Further examination of the inhibitory effect, the inability of certain strains to form conidial colonies on sucrose-sorbose-agar medium, revealed that many experimental conditions were capable of altering the colony-forming efficiency of the mutant strain. As long as sucrose, sorbose, and agar were present in the specified proportions and all autoclaved together, the mutant inhibitory effect could not be relieved. Relief of inhibition could be accomplished by raising the sucrose level to one percent, decreasing sorbose to 0.25 percent, or decreasing agar to zero. Also, separate autoclaving of the agar from salts-sucrose-sorbose followed by combining of the two after autoclaving was effective in relieving inhibition. A component of the agar which was water-soluble and dialyzable and which could be isolated by

chromatographic procedures was also shown to effect inhibition, in much the same manner as whole agar. Heparin, which like agar is a sulfated polysaccharide, was found to simulate the agar effect, as were other substances such as sodium citrate and ammonium tartrate characterized by their common possession of carboxyl end-groups.

The fact that whenever glucose was added to the medium inhibition was relieved suggests that the inhibitory effect is due to the non-availability of sufficient carbon sources from sucrose to support mutant colony-formation. However, when sucrose-sorbose-agar medium was inoculated with conidia of the mutant type which had germinated and begun to grow in liquid medium, these germinated conidia were capable of continuing growth on the inhibitory medium to form colonies. Such findings cannot be adequately explained by the non-availability postulate, but suggest that the inhibitory effect is due to the presence of a finite amount of an inhibitory substance, and that the increased surface area of germinated conidia exposed to this substance results in certain growing sites' retaining their growth capability, and utilizing carbon sources which are available in the sucrose-sorbose-agar medium to continue growth. Additional experiments designed to distinguish between these two hypotheses have failed to produce sufficient evidence to warrant a clear-cut decision as to whether the inhibition is a result of non-availability of sugars or a result of the formation of a separate inhibitory substance.

The mutant phenotype is expressed only under conditions

imposed by the presence of sorbose in the medium. Sorbose is known to induce colony formation by a structural alteration of the cell wall. Therefore it seems probable that the mutant phenotype is an expression of the interaction between the inability of the cell wall to function normally and the presence of sucrose and agar in the medium.

The genetic basis of the mutant phenotype has been established. In certain crosses, the progeny segregate one to one for mutant and normal types, indicating a single gene difference. Analysis of the progeny of other crosses suggests the presence of other genetic factors which may interact with the mutant gene to distort the segregation pattern.